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Androgen Receptor Splice Variants Activate Androgen Receptor Target Genes and Support Aberrant Prostate Cancer Cell Growth Independent of Canonical Androgen Receptor Nuclear Localization Signal*[S]

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Background: Truncated AR splice variants support castration-resistant prostate cancer.
Results: The AR NTD/DBD core is sufficient for AR variants to access the nucleus, activate AR target genes, and support androgen-independent prostate cancer cell growth.
Conclusion: Diverse truncated AR variants are constitutively active transcription factors.
Significance: These novel biochemical properties could lead to the development of new prostate cancer therapies.

Synthesis of truncated androgen receptor (AR) splice variants has emerged as an important mechanism of prostate cancer (PCa) resistance to AR-targeted therapy and progression to a lethal castration-resistant phenotype. However, the precise role of these factors at this stage of the disease is not clear due to loss of multiple COOH-terminal AR protein domains, including the canonical nuclear localization signal (NLS) in the AR hinge region. Despite loss of this NLS, we show that diverse truncated AR variant species have a basal level of nuclear localization sufficient for ligand-independent transcriptional activity. Whereas full-length AR requires Hsp90 and importin-β for active nuclear translocation, basal nuclear localization of truncated AR variants is independent of these classical signals. For a subset of truncated AR variants, this basal level of nuclear import can be augmented by unique COOH-terminal sequences that reconstitute classical AR NLS activity. However, this property is separable from ligand-independent transcriptional activity. Therefore, the AR splice variant core consisting of the AR NH_2-terminal domain and DNA binding domain is sufficient for nuclear localization and androgen-independent transcriptional activation of endogenous AR target genes. Indeed, we show that truncated AR variants with nuclear as well as nuclear/cytoplasmic localization patterns can drive androgen-independent growth of PCa cells. Together, our data demonstrate that diverse truncated AR species with varying efficiencies of nuclear localization can contribute to castration-resistant PCa pathology by driving persistent ligand-independent AR transcriptional activity.

The androgen receptor (AR)[2] is a modular steroid receptor transcription factor, composed of a large, disordered NH_2-terminal domain (NTD), a central DNA binding domain (DBD), and a COOH-terminal ligand binding domain (LBD) and transcriptional activation function-2 coactivator binding interface (1). Androgen depletion therapy (ADT) for locally advanced or metastatic prostate cancer (PCa) relies on blocking androgen production and/or androgen binding to the AR LBD. However, most PCa mortality is due to the development of castration-resistant PCa (CRPCa), which is driven by pathologic reactivation of the AR during ADT (2). An ongoing AR dependence of CRPCa supports the concept that AR is a lineage survival factor in prostate cells (3), and this knowledge has led to the development of additional strategies for AR inhibition, including the CYP17 inhibitor abiraterone, which extends lifespan in a subset of CRPCa patients by further suppressing androgen production in the adrenal cortex as well as tumor tissue (4).

One possible mechanism of aberrant AR reactivation during ADT is the synthesis of COOH-terminal truncated AR variants lacking the AR LBD and activation function-2 domain (5). These AR variants arise through alterations in splicing and encode protein products composed of the AR NTD/DBD core and novel COOH-terminal extensions of variable length and sequence (6–10). Antibodies have been developed for the COOH-terminal extension of the truncated AR-V7 variant (also referred to as AR3), demonstrating that expression levels increase concomitant with stage of hormonal progression (7). Moreover, increased expression of AR-V7 in hormone-naïve prostatectomy specimens is associated with disease relapse

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[S] This article contains supplemental Experimental Procedures, Table 1, and Figs. 1–8.

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[2] The abbreviations used are: AR, androgen receptor; NTD, NH_2-terminal domain; DBD, DNA binding domain; LBD, ligand binding domain; ADT, androgen depletion therapy; PCa, prostate cancer; CRPCa, castration-resistant PCa; NLS, nuclear localization signal; ARE, androgen response element; LUC, luciferase.

after surgery (7). In surgical specimens of skeletal CRPCa metastases, levels of AR-V7 and AR-V1 mRNA are inversely correlated with survival (11). These studies indicate that changes in AR splicing are important for clinical CRPCa progression and may drive resistance to therapies that require an intact AR LBD, including next-generation inhibitors such as abiraterone (12).

Currently, it is unclear whether diverse truncated AR variants can activate the AR transcriptional program because many of these species are predicted to have lost the canonical AR nuclear localization signal (NLS) in the exon 4-encoded hinge region. Similar to other steroid receptors, AR transcriptional activity is tightly regulated by ligand binding. In the unbound, inactive state, the AR is a predominantly cytoplasmic protein bound by heat shock family chaperone proteins and immunophilins (13). AR nuclear import proceeds via a classical pathway wherein nuclear translocation is initiated by ligand binding, which exposes the bipartite AR NLS and allows binding to the importin-α adapter protein and importin-β carrier protein, translocation through the nuclear core complex, and Ran-dependent release into the nucleus (14, 15). Because domains required for interaction with these pathways appear to be lost, it has been suggested that many truncated AR variants may have exclusively cytoplasmic functions or even function as dominant negative species (7, 9, 10). Therefore, how these diverse species may be involved in CRPCa progression is not clear. To address this, we investigated the biochemical properties of individual truncated AR variants with the goal of identifying general molecular features that could be exploited for developing new CRPCa therapies.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—LNCaP, 22Rv1, DU145, 293T, and Cos-7 cells were obtained from American Type Culture Collection (ATCC). LNCaP, 22Rv1, and DU145 cells were maintained in RMPI 1640 (Invitrogen) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37 °C. The 293T and Cos-7 cell lines were cultured in DMEM with 10% FBS. For androgen response experiments, cells were seeded in 10% charcoal-stripped serum for 48 h. Cells were then stimulated for 24 h by replacement of growth medium with medium containing 1 nM mibolerone (Biomol) or dihydrotestosterone (Sigma).

Antibodies and siRNA Reagents—Small interfering RNAs (targeted to AR exons 1, 3, and 7) were purchased from Dharmacon. Primary antibodies specific for the AR NTD (N-20, catalogue #sc-816; A-441, catalogue #sc-7305), ERK-2 (D-2, catalogue #sc-1647), HA tag (F-7, catalogue #sc-7392), and α-tubulin (catalogue #sc-23948) were purchased from Santa Cruz Biotechnology. An antibody specific for lamin A/C (4C11, catalogue #4777) was purchased from Cell Signaling.

Plasmids—Plasmid constructs harboring full-length AR (p5HBhAR-A), AR 1/2/3/2b, MMTV-LUC, −5746 PSA-LUC, and 4XARE-E4-LUC have been described (6, 16). Construction details for plasmid and lentiviral reagents used in this study are provided in the supplemental Experimental Procedures.

Luciferase Reporter Gene Assays—LNCaP cells were transfected by electroporation exactly as described (6, 17). DU145 cells were transfected using Superfect reagent (Qiagen) exactly as described (6). For all reporter-based experiments, 24–48 h post-transfection cells were re-fed with serum-free medium containing 1 nM mibolerone or 0.1% ethanol as vehicle control for 24 h. Cells were harvested in 1× passive lysis buffer provided in a Dual Luciferase Assay kit (Promega). Activities of the firefly and *Renilla* luciferase reporters were assayed using a Dual Luciferase Assay kit as per the manufacturer's recommendations. Transfection efficiency was normalized by dividing firefly luciferase activity by *Renilla* luciferase activity. Data presented represent the mean ± S.E. from at least three independent experiments, each performed in triplicate.

Subcellular Fractionation—LNCaP cells were transfected under androgen-free conditions with expression vectors encoding truncated AR variants using PolyExpress (Excellgen) exactly as per the manufacturer's protocol. 22Rv1 cells were electroporated under androgen-free conditions with siRNA targeted to AR exon 7 (Dharmacon) as described (6). Transfected cells were cultured 24 h post-transfection and then treated for 24 h with medium containing 1 nM mibolerone or 0.1% ethanol. After treatment, transfected cells were washed in 1× phosphate-buffered saline (PBS), harvested in hypotonic buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and a 1× final concentration of Roche Applied Science Mini complete protease inhibitor), and incubated for 20 min on ice. Cells were lysed by 10 passages through 25-gauge needles. The cytosolic fraction (supernatant) was collected by centrifugation at 720 × *g*, 5 min, 4 °C. The nuclear pellet was washed twice by resuspending in 500 μl of hypotonic buffer followed by 10 passages through 25-gauge needles. Nuclei were pelleted by centrifugation at 3000 × *g* for 10 min at 4 °C. Isolated cytosolic and nuclear fractions were resuspended in 1× Laemmli buffer (18), boiled for 5 min, and then loaded equally by volume for Western blot analysis.

Western Blot—Cell lysates in 1× Laemmli buffer were subjected to Western blot analysis as described (6). Blots were incubated with primary antibodies overnight at 4 °C and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h. Blots were developed by incubation with Super Signal chemiluminescence reagent (Pierce) and exposed to film.

Immunofluorescence—Cos-7, LNCaP, and DU145 cells were seeded in complete medium on coverslips the day before transfection. Cells were transfected under androgen-free conditions with 1 μg of expression vector encoding full-length AR or truncated AR variants using Lipofectamine 2000 (Invitrogen) for Cos-7 cells, electroporation for LNCaP cells, or Superfect (Qiagen) for DU145 cells. The next day culture medium was replaced with serum-free medium containing 1 nM mibolerone or 0.1% ethanol. Cells were maintained an additional 24 h and then fixed with ice-cold methanol for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature, and blocked with 1× PBS containing 10% FBS for 1 h at room temperature. Cells were incubated overnight at 4 °C with AR-N20 diluted 1:1000 or HA-F7 diluted 1:500 in PBS. Unbound primary antibody was removed by three washes with PBS, and then cells were incubated with fluorescein isothiocya-

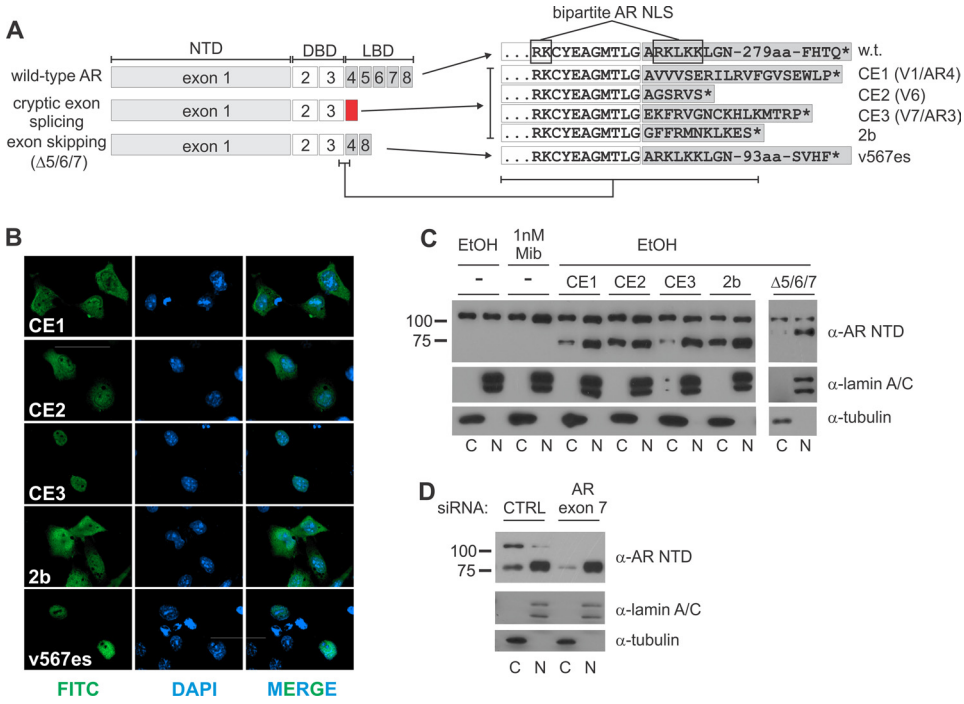


FIGURE 1. **Constitutive nuclear localization of truncated AR variants with diverse COOH-terminal extensions.** *A*, shown is a schematic of COOH-terminal tails of truncated AR variants aligned with the AR hinge region. The multiple names that have been assigned to several of these variants are indicated (7, 8). *B*, Cos-7 cells expressing truncated AR variants 1/2/3/CE1, 1/2/3/CE2, 1/2/3/CE3, 1/2/3/2b, or v567es were stained with an antibody specific for the AR NTD (FITC signal), and nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy. Representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *C*, LNCaP cells expressing truncated AR variants were cultured in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (*EtOH*, vehicle control). Cell lysates were separated into nuclear (*N*) and cytoplasmic (*C*) fractions and analyzed by Western blot with antibodies specific for the AR NTD, lamin A/C (nuclear marker), and tubulin (cytoplasmic marker). *D*, 22Rv1 cells were electroporated with non-targeted siRNA (*CTRL*) or siRNA targeted to AR Exon 7. Cells were cultured, fractionated, and analyzed by Western blot exactly as described in *C*.

nate (FITC)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) at a 1:500 dilution in PBS containing 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 1 h at room temperature. Slides were washed with PBS and mounted with Mowoil (Calbiochem), and images were captured using a confocal laser scanning microscope (Olympus Fluoview FV500) equipped with a 40× objective. To optimize microscopy data for print media, hue adjustments were made to all immunofluorescence images (*red*, +90; *green*, +90; *blue*, −90) using CorelDraw software.

Lentivirus Packaging and Transduction—Lentivirus expressing GFP, AR 1/2/3/CE3, 1/2/3/CE3-R617A/K618A, 1/2/3/CE3-K629A/R631A, AR 1–627aa, AR 1/2/3/2b, and AR v567es was prepared using a standard third generation packaging system in 293T cells. Briefly, 293T cells were cotransfected with lentivirus vectors and packaging vectors pCMVΔR8.91 (19) and pMD.G (20) at a ratio of 4:3:1 using Lipofectamine 2000. Medium containing lentivirus was collected from 12 to 96 h post-transfection and concentrated using the Lenti-X Concentrator (Clontech). Titers for LNCaP transductions were tested and normalized to achieve protein expression levels equivalent to endogenous AR.

Total RNA Extraction and Quantitative Real Time RT-PCR—Total RNA was isolated from LNCaP cells using TRIzol (Invitrogen). RNA was reverse-transcribed using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science) following the manufacturer's instructions. cDNA was subjected to quantitative PCR using PerfeCTa SYBR Green FastMix (Quanta Biosciences) with primer sets specific for PSA, hK2, TMPRSS2, FKBP51, and GAPDH (see supplemental Table 1). -Fold change in mRNA expression levels was calculated by the comparative Ct method using the formula $2^{-(\Delta\Delta Ct)}$ and GAPDH as calibrator as described (6).

Analysis of Cell Growth by Crystal Violet Mitogenic Assay—Lentivirus-transduced LNCaP cells were seeded at equal density on 24-well plates. Lentivirus-transduced 22Rv1 cells were electroporated with small interference RNA (siRNA) targeted to AR exon 1 as described (6, 17) and seeded at equal density on 24-well plates. At the indicated time points, cells were fixed and stained with crystal violet as described (21).

RESULTS

Truncated AR Variants with Diverse COOH-terminal Extensions Are Constitutively Nuclear Transcription Factors—The majority of AR transcriptional activity is mediated by the AR NTD and deletion of the AR LBD results in constitutive, ligand-independent activity (22). However, early biochemical studies that established these fundamental principles employed AR fragments that retained the bipartite NLS in the AR hinge region. Most truncated AR variants expressed in CRPCa have a disrupted bipartite NLS due to altered splicing (Fig. 1*A*). Therefore, it has been proposed that many of these species would not be able to access the nucleus, which is a prerequisite for transcriptional activation. However, this theory has not been rigorously tested. Therefore, we transfected cells with diverse truncated AR variants and performed immunofluorescence

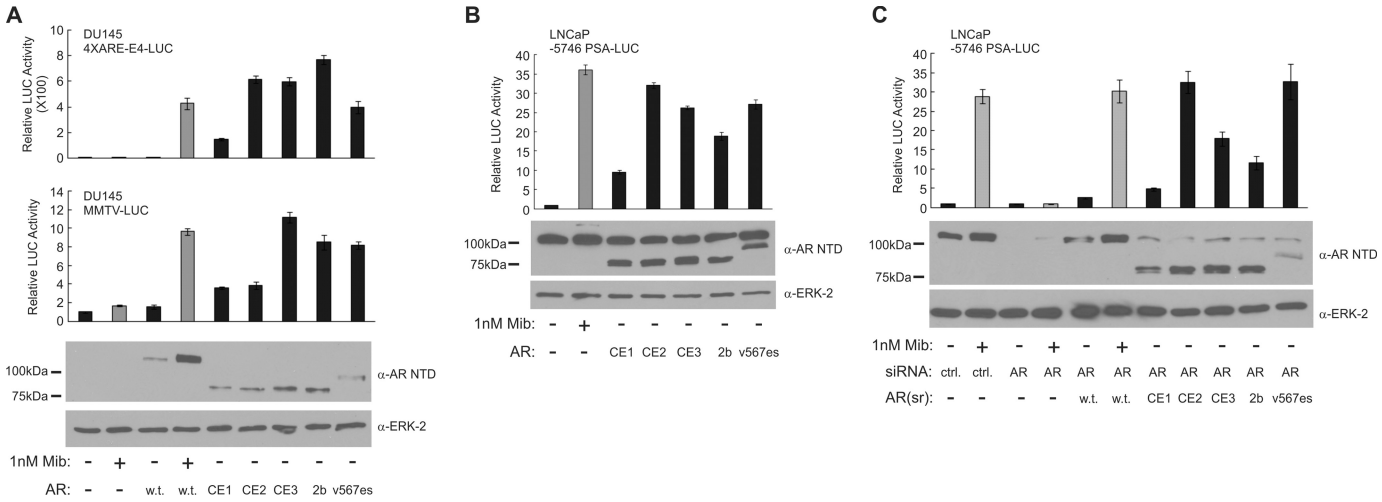


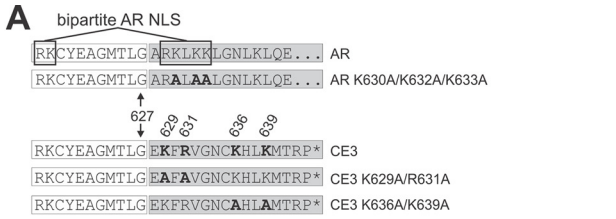
FIGURE 2. **Constitutive transcriptional activity of truncated AR variants with diverse COOH-terminal extensions.** *A*, DU145 cells were transiently transfected with 4XARE-E4-LUC or MMTV-LUC reporters and expression plasmids encoding full-length AR or truncated variants 1/2/3/CE1, 1/2/3/CE2, 1/2/3/CE3, 1/2/3/2b, and v567es. Cells were cultured in serum-free medium containing 1 nM mibolerone (*Mib*) or vehicle control (ethanol). Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent mean ± S.E. from at least three independent experiments, each performed in duplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *B*, LNCaP cells were transiently transfected with a −5746 PSA-LUC reporter and truncated AR variants and analyzed by luciferase assay and Western blot as in *A*. *C*, LNCaP cells were transiently transfected and analyzed exactly as described in *B* with the exception of the use of AR exon 7-targeted siRNA and siRNA-resistant AR expression constructs (denoted *sr* (17)).

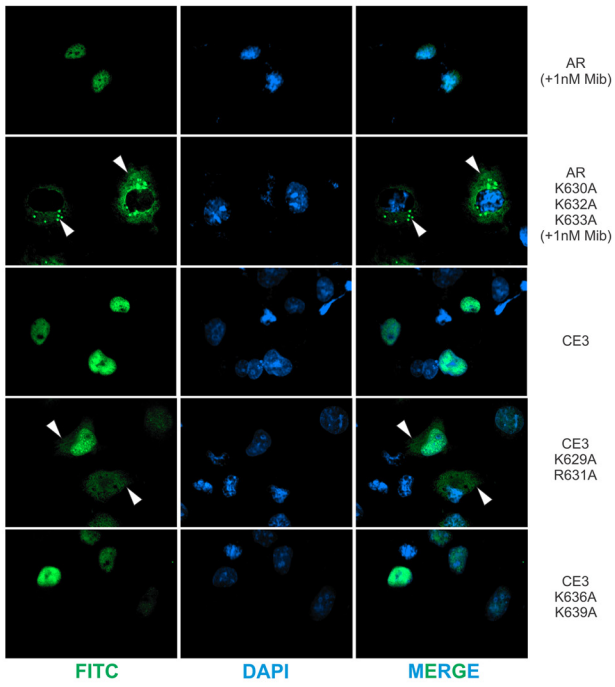
microscopy (Fig. 1*B* and see supplemental Fig. 1) and biochemical fractionation (Fig. 1*C*). Truncated AR variants displayed significant differences in predominance of nuclear localization, but all were able to access the nucleus. Nuclear localization patterns were the same in AR-null Cos-7 and DU145 cells and AR-dependent LNCaP cells, indicating that nuclear localization is not affected by cell type or presence of full-length AR (Fig. 1, *B* and *C*, and see supplemental Fig. 1). To test this directly, we selectively knocked-down full-length AR expression in 22Rv1 cells using siRNA targeted to AR exon 7 and observed no effect on the nuclear localization of endogenous truncated AR variants (Fig. 1*D*). Therefore, truncated AR variants with the AR NTD/DBD core and diverse COOH-terminal extensions are nuclear proteins.

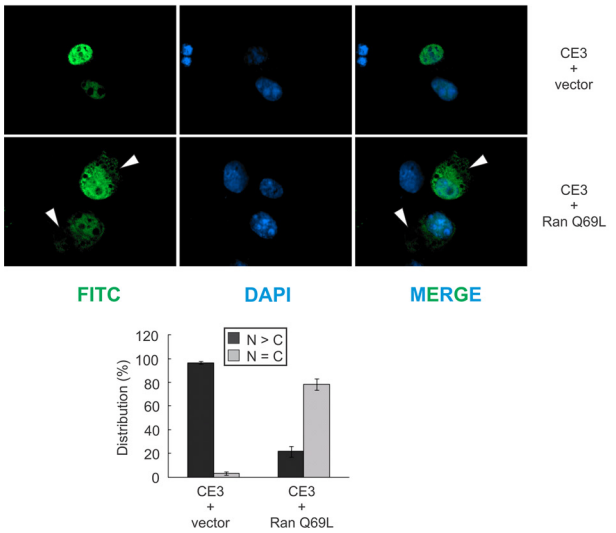
To test whether the observed differences in nuclear localization between truncated AR variants underlie differences in transcriptional activity, we performed transient transfection experiments in AR-null DU145 PCa cells and AR-dependent LNCaP cells (Fig. 2, *A* and *B*, and see supplemental Fig. 2*A*). Different truncated AR variants displayed varying strengths of transcriptional activity in a promoter-dependent but cell line-independent manner. To test a requirement for full-length AR, we performed knockdown/re-expression experiments in LNCaP cells. Knockdown of full-length AR completely inhibited ligand-dependent promoter activity, and re-expression of siRNA-resistant full-length AR restored this ligand-dependent activity. Conversely, re-expression of individual siRNA-resistant truncated AR variants was able to restore promoter activity in the absence of ligand (Fig. 2*C* and see supplemental Fig. 2*B*). The levels of transcriptional activity in these assays were nearly identical to the levels observed in the absence of knock-down (Fig. 2, *B* and *C*, and see supplemental Fig. 2), confirming that full-length AR is not required for this activity.

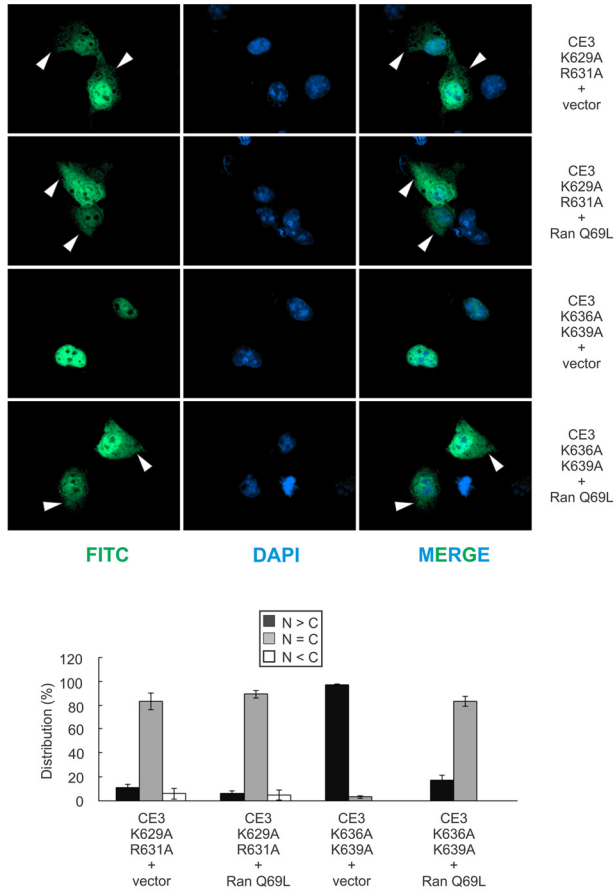
Reconstitution of Bipartite AR NLS Enhances Truncated AR Variant Nuclear Localization but Not Transcriptional Activity—Despite the observation that individual truncated AR variants exhibited varying degrees of transcriptional activity on AR-responsive promoters, there did not appear to be a perfect relationship between efficiency of nuclear localization and transcriptional activity. This suggests that nuclear localization is not the primary determinant of AR variant transcriptional activity. To test this concept, we focused on the AR-V7/AR3 variant generated by splicing of AR exons 1/2/3/CE3, which displayed strong nuclear localization and high transcriptional activity. This variant contains two clusters of basic amino acids in the COOH-terminal tail encoded by AR exon CE3, one of which aligns with the second basic amino acid cluster of the wild-type AR bipartite NLS (Fig. 3*A*). Consistent with previous reports (15, 23), a K630A/K632A/K633A compound mutation blocked ligand-induced nuclear localization of full-length AR (Fig. 3*B*). Alanine mutation of Lys-629 and Arg-631 in the truncated AR 1/2/3/CE3 variant, which both align to the second basic amino acid cluster in the full-length AR NLS, shifted 1/2/3/CE3 expression from predominantly nuclear to a mixed nuclear/cytoplasmic pattern (Fig. 3*B* and see supplemental Fig. 3). Conversely, alanine mutation of Lys-636 and Lys-639 in the truncated AR 1/2/3/CE3 variant had no effect on nuclear localization (Fig. 3*B*). We next asked whether dominant negative Ran Q69L, which prevents the carrier importin-β from releasing cargo into the nucleus (24), could alter subcellular distribution of the truncated AR1/2/3/CE3 variant. Indeed, dominant negative Ran Q69L caused a shift in AR 1/2/3/CE3 expression to a mixed nuclear/cytoplasmic pattern (Fig. 3*C*). A similar effect of dominant negative Ran Q69L was observed on the nuclear K636A/K639A mutant version of AR 1/2/3/CE3, but no effect was observed for the nuclear/cytoplasmic K629A/R631A mutant version, showing that the dominant negative effects of

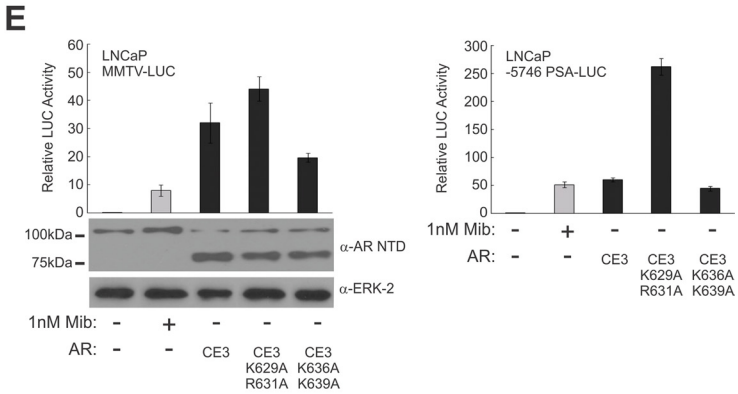
Ran Q69L were mediated through the Lys-629/Arg-631 motif (Fig. 3*D*). These data indicate that AR 1/2/3/CE3 displays enhanced nuclear localization because amino acid residues Lys-629 and Arg-631 reconstitute the second half of the bipartite AR NLS. Notably, alanine mutations in these residues did not have the same magnitude of effect as for full-length AR, indicating alternate modes of nuclear import existed for truncated AR variants. Indeed, the K629A/R631A mutant version of AR 1/2/3/CE3 displayed a paradoxical higher level of transcriptional activity on various AR-responsive promoters in AR-dependent LNCaP cells and AR-null DU145 cells, further confirming that the classical mode of AR nuclear import is not the











main determinant of truncated AR variant activity (Fig. 3*E* and see supplemental Fig. 4).

To test this concept more rigorously, we asked whether increasing the basic amino acid content in the short COOH-terminal tail of the truncated AR 1/2/3/CE2 variant would enhance nuclear localization by reconstituting bipartite AR NLS function. Alignment of the COOH-terminal tails of AR 1/2/3/CE2 and AR 1/2/3/CE3 revealed that they both harbor arginine at position 631, but AR 1/2/3/CE2 harbors glycine at position 629 (Fig. 4*A*). As predicted, generation of a G629K mutant, which mimics the basic amino acid cluster in the AR 1/2/3/CE3 COOH-terminal tail, led to enhanced nuclear localization (Fig. 4*B*) that was reversed by dominant negative Ran Q69L (Fig. 4*C*). However, despite this enhanced nuclear localization, transcriptional activity on an AR-responsive promoter was unchanged (Fig. 4*D*).

These data indicate that truncated AR variants possess an intrinsic, basal level of nuclear localization that may be supported by a pathway(s) different from that required by full-length AR. This may be due to unmasking of additional regulatory signals or loss of the strong COOH-terminal AR nuclear export sequence by truncation of the AR LBD (25). Indeed, even nuclear localization of the AR v567es variant, which retains the AR exon 3/4 splice junction and wild-type AR NLS sequence, was only modestly affected by dominant negative Ran Q69L (see supplemental Fig. 5). We, therefore, queried whether activity of the Hsp90 chaperone complex, which is required upstream of importin-α/β for AR ligand binding and translocation to the nucleus (13), was required for nuclear access of truncated AR variants. A complete block in androgen-induced nuclear import of full-length AR was observed after treatment of cells with the Hsp90 inhibitor, 17-*N*-allylamino-17-demethoxygeldanamycin (Fig. 5*A*), but there was no effect of this inhibitor on constitutive nuclear localization of the truncated AR 1/2/3/CE3 and v567es variants (Fig. 5, *B* and *C*). These data thus confirm that the canonical pathways required for nuclear import of full-length AR are not required for nuclear import of truncated AR variants.

AR NTD Encoded by Exon 1 and AR DBD Encoded by Exons 2 and 3 Are Sufficient for Nuclear Localization and Transcriptional Activity of Truncated AR Variants—We next turned our attention to the role of the first basic amino acid cluster in the bipartite AR NLS, which is encoded by AR exon 3. Even though the AR NLS/importin-α crystal structure did not reveal a direct binding role for this part of the NLS (15), we postulated that this region may be able to compensate in the absence of the second, main cluster of basic amino acids. Therefore, we generated R617A/K618A compound mutations in full-length AR and truncated AR variants (Fig. 6*A*). Interestingly, this compound mutation did not inhibit ligand-dependent transcriptional activity or nuclear localization of full-length AR (Fig. 6, *B* and *C*), but this mutation impaired transcriptional activity of truncated AR variants (Fig. 6*D*). The R617A/K618A compound mutant version of the truncated AR 1/2/3/CE3 variant, but not the 1/2/3/CE2 variant, displayed impairment in nuclear access (Fig. 6*E* and see supplemental Fig. 6), indicating that this motif plays a minor role in truncated AR variant nuclear localization. More significantly, these data suggested that the intrinsic, basal level of nuclear localization displayed by truncated AR variants proceeds in a manner completely independent of either basic amino acid cluster in the bipartite AR NLS. To test this idea directly, we generated a R617A/K618A/K629A/R631A compound mutant of the truncated AR 1/2/3/CE3 variant to neutralize both basic amino acid clusters (Fig. 7*A*). This compound mutant version of the truncated AR 1/2/3/CE3 variant displayed roughly equal distribution in the nucleus and cytoplasm, demonstrating that basal nuclear localization of truncated AR variants could proceed independently from the canonical AR NLS (Fig. 7*B*). However, this compound mutant was transcriptionally inactive (Fig. 7, *C* and *D*), illustrating that these residues in the second α-helix of the AR DBD are important for transcriptional function.

To probe the requirement for this first cluster of basic amino acids in more detail, we generated a synthetic truncated AR variant composed of only the exon 1-encoded NTD and exon 2/3-encoded DBD (AR amino acids 1–627) as well as a R617A/K618A compound mutant version of this synthetic AR variant (Fig. 7*A*). The synthetic AR 1–627 variant displayed approximately equal expression in the nucleus and cytoplasm (Fig. 7*B*) as well as strong ligand-independent transcriptional activity on AR-responsive promoters (Fig. 7, *C* and *D*). This finding confirms that COOH-terminal tail sequences encoded by exons spliced after exon 3 are not required for transcriptional function. However, the R617A/K618A compound mutant version of the synthetic AR 1–627 variant was transcriptionally inactive (Fig. 7, *C* and *D*). This compound mutant was still able to access the nucleus, but a pattern consistent with protein aggregation was also noted (Fig. 7*B*). These data further implicate a unique and important role for exon 3-encoded Arg-617 and Lys-618 in structural integrity of the AR DBD in the context of truncated AR variants but not full-length AR.

FIGURE 3. **The COOH-terminal tail encoded by AR exon CE3 reconstitutes the canonical AR NLS.** *A*, shown is a schematic of alanine substitutions in full-length AR and the truncated AR 1/2/3/CE3 variant. *B*, Cos-7 cells expressing parental and mutant plasmids illustrated in *A* were maintained in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). Increased cytoplasmic localization is denoted by *white arrows*. *C*, Cos-7 cells were transfected with an AR 1/2/3/CE3 expression plasmid and dominant negative Ran Q69L. Cells were stained and subjected to confocal microscopy as in *A*. Increased cytoplasmic localization is denoted by *white arrows*. Quantification of predominantly nuclear ($N > C$) *versus* equal nuclear and cytoplasmic ($n = C$) expression is shown at the *bottom*. Bars represent the mean ± S.D. of 100 cells scored in three independent experiments. *D*, Cos-7 cells were transfected with mutant plasmids indicated in *A* along with dominant-negative RanQ69L (or vector control). Cells were stained and subjected to confocal microscopy as in *A*. Increased cytoplasmic localization is denoted by *white arrows*. Quantification of predominantly nuclear ($N > C$), equal nuclear and cytoplasmic ($n = C$) or predominantly cytoplasmic ($N < C$) expression is shown at the *bottom*. *Bars* represent mean ± S.D. of 100 cells scored in three independent experiments. *E*, LNCaP cells were transiently transfected with MMTV-LUC or −5746-LUC reporters and parental and mutant plasmid constructs depicted in *A*. Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent the mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1.

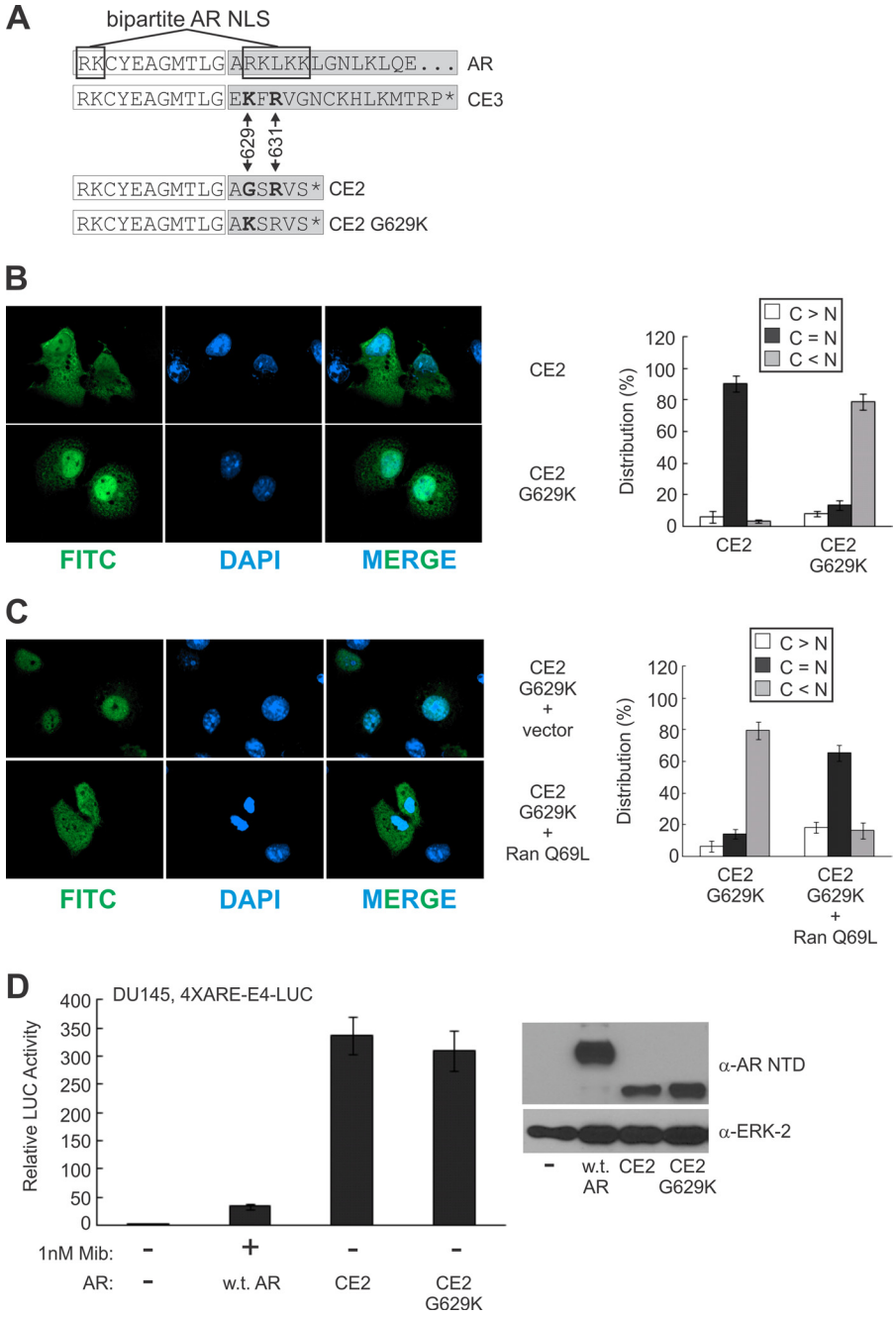


FIGURE 4. **NLS reconstitution does not enhance transcriptional activity of the truncated AR 1/2/3/CE2 variant.** *A*, shown is alignment of full-length AR, AR 1/2/3/CE3, and AR 1/2/3/CE2 and location of the G629K mutation. *B*, *left*, Cos-7 cells expressing 1/2/3/CE2 and the G629K mutant were maintained in serum-free medium and stained with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *Right*, shown is quantification of predominantly nuclear ($N > C$), equally distributed nuclear and cytoplasmic ($n = C$), or predominantly cytoplasmic ($N < C$) expression. Bars represent mean ± S.D. of 100 cells scored in 3 independent experiments. *C*, *left*, Cos-7 cells were transfected as in *B* with the addition of dominant-negative RanQ69L (or vector control). *Right*, shown is quantification of predominantly nuclear ($N > C$), equally distributed nuclear and cytoplasmic ($n = C$), or predominantly cytoplasmic ($N < C$) expression. *Bars* represent mean ± S.D. of 100 cells scored in 3 independent experiments. *D*, *left*, DU145 cells were transfected with a 4XARE-E4-LUC reporter and parental/mutant plasmid constructs depicted in *A*. *Bars* represent mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *Right*, cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control).

AR NTD/DBD Fragment Is Sufficient for Constitutive, Ligand-independent Transcriptional Activation of AR Target Genes—To determine whether these biochemical principles applied to endogenous AR target genes, we generated lentivirus encoding GFP, AR 1/2/3/CE3, AR 1/2/3/CE3 R617A/K618A, AR 1/2/3/CE3 K629A/R631A, or the synthetic AR 1–627 variant (Fig. 8*A*). We chose to study transcriptional responses of PSA, hK2, TMPRSS2, and FKBP51 because promoters and enhancers of these genes are regulated by a variety of classical and selective androgen response elements (AREs) that may reflect differential requirements for AR COOH-terminal extensions (26). Infection of LNCaP cells with lentivirus encod-

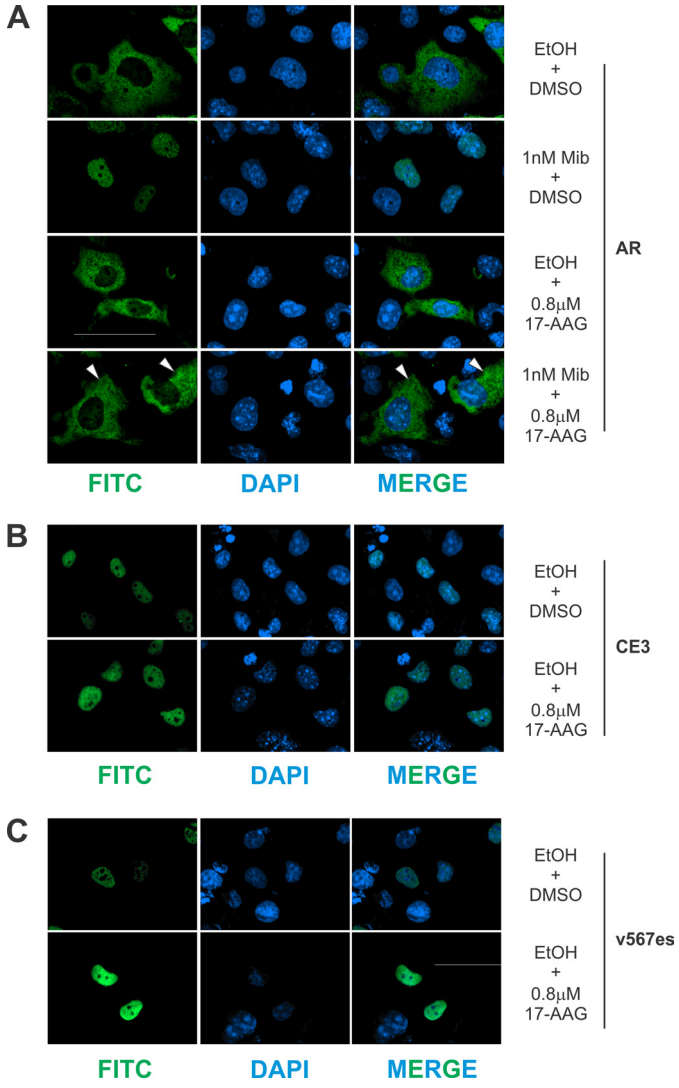


FIGURE 5. **Truncated AR variants do not require Hsp90 activity for nuclear localization.** Cos-7 cells expressing full-length AR (*A*), AR 1/2/3/CE3 (*B*), or AR v567es (*C*) were maintained in serum-free medium containing the indicated combinations of 1 nM mibolerone (*Mib*, synthetic androgen), ethanol (*EtOH*, vehicle control), 0.8 μM 17-*N*-allylamino-17-demethoxygeldanamycin (*17-AAG*, Hsp90 inhibitor), and dimetylsulfoxide (*DMSO*, vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *White arrowheads* are shown to highlight cytoplasmic expression caused by 17-*N*-allylamino-17-demethoxygeldanamycin.

ing the truncated AR 1/2/3/CE3 variant elicited androgen-independent transcriptional activation of these AR target genes (Fig. 8*B*). Inactivation of the reconstituted AR 1/2/3/CE3 NLS by K629A/R631A mutations or truncation after position 627 still resulted in robust, ligand-independent transcriptional activation of the same AR target genes (Fig. 8*B*). However, inactivation of the Arg-617/Lys-618 motif in the second α-helix of the AR DBD of AR 1/2/3/CE3 impaired transcriptional activation of PSA, hK2, and FKBP51 target genes. Noteworthy, the TMPRSS2 gene did not display impairment in androgen-independent expression levels in response to any of these mutations in the AR DBD or COOH-terminal tail (Fig. 8*B*). Together, these data illustrate a new paradigm wherein the AR NTD/DBD core encoded by exons 1, 2, and 3 are sufficient for a basal level of access to the nucleus, engagement with endogenous AR target genes, and efficient transcriptional activation of these target genes in an androgen-free environment. Because CRPCa is characterized by persistent AR transcriptional activity under conditions of ADT, these findings indicate that AR variants consisting of the AR NTD and DBD may contribute to disease progression regardless of the identity of their COOH-terminal extensions.

AR 1/2/3/2b Protein Is Expressed in 22Rv1 Cells and Can Support Androgen-independent Growth of Prostate Cancer Cells—The CRPCa 22Rv1 cell line displays altered AR splicing patterns as a result of a 35-kb intragenic AR tandem duplication encompassing AR exon 3 (27, 28). We and others have demonstrated that 22Rv1 cells express two separate mRNAs harboring cryptic exon 2b composed of contiguously spliced AR exons 1/2/2b or AR exons 1/2/3/2b. The AR 1/2/2b variant is different from other truncated AR variants that have been reported because it encodes the NTD and first zinc finger of the AR DBD (see supplemental Fig. 7*A*). Because there is concern that many of the variant AR mRNAs that have been identified in 22Rv1 and other models may simply be spurious splicing byproducts that are degraded by nonsense-mediated RNA decay (29), we asked whether these protein species are expressed in 22Rv1 cells. To test for AR 1/2/2b expression, we first employed siRNA specific for AR exon 3, which would not target the AR 1/2/2b isoform. Transfection of 22Rv1 cells with this siRNA led to complete ablation of all truncated AR variant protein expression, indicating that AR 1/2/2b is not a protein constituent in these cells (see supplemental Fig. 7*B*). Next, we developed polyclonal antibodies specific for the COOH-terminal extension encoded by AR exon 2b. Crude antisera as well as antibodies purified on an immobilized peptide affinity column were able to immunoprecipitate an ~80-kDa AR polypeptide from 22Rv1 lysates (see supplemental Fig. 7*C*). Moreover, affinity-purified exon 2b polyclonal antibodies recognized a truncated AR variant species in Western blot experiments that was the same size as ectopic AR 1/2/3/2b protein (see supplemental Fig. 7*D*). Therefore, we conclude that AR 1/2/3/2b is expressed as a mature protein species in 22Rv1 cells.

The predominantly nuclear AR 1/2/3/CE3 variant (referred to as AR-V7 or AR3) is also expressed as a mature protein in 22Rv1 cells and can induce androgen-independent growth when expressed in androgen-dependent LNCaP cells (7, 8). In subcellular localization assays, AR 1/2/3/2b protein displayed a mixed cytoplasmic/nuclear localization pattern (Fig. 1, *B* and *C*). Therefore, we chose to employ the AR 1/2/3/2b variant to determine whether truncated AR species with partial nuclear localization could also support androgen-independent growth of PCa cells. We infected LNCaP cells with lentivirus encoding AR 1/2/3/2b, AR 1/2/3/CE3, or AR v567es and found that each of these truncated AR variants could induce an androgen-independent growth phenotype (Fig. 9*A*). These data confirm that classical NLS activity is independent from truncated AR variant function in PCa cells. Intriguingly, during the course of these studies we noted variability in the magnitude of androgen-independent growth that each AR variant induced in LNCaP cells. Paradoxically, we noted that higher levels of truncated AR variant expression consistently resulted in lower levels of

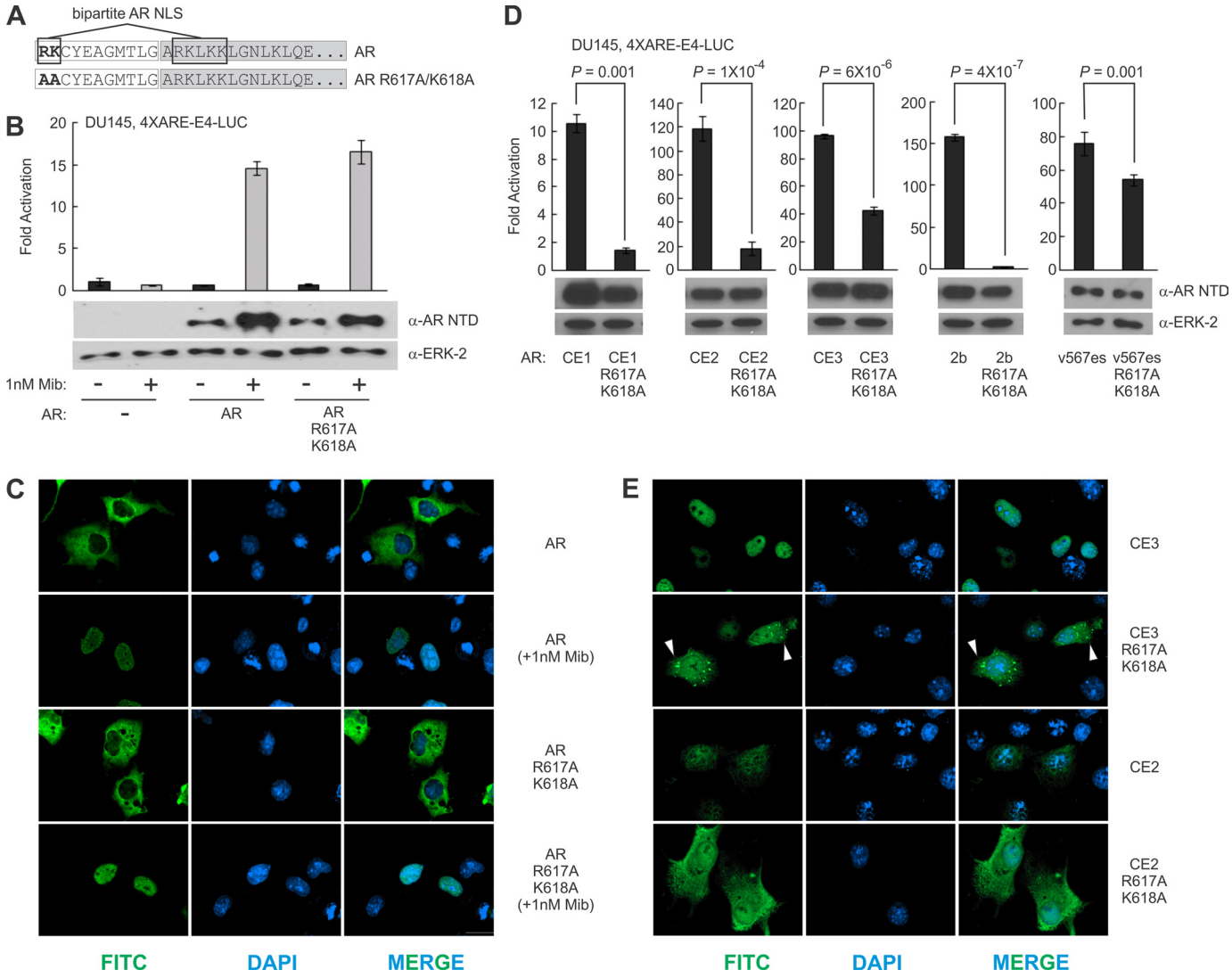


FIGURE 6. **A unique requirement for the Arg-617/Lys-618 motif in the AR DBD terminal helix.** *A*, shown is a schematic of the AR hinge region and location of the Arg-617/Lys-618 motif. *B*, DU145 cells were transiently transfected with 4XARE-E4-LUC and full-length AR or a R617A/K618A mutant. Cells were cultured in serum-free medium containing 1 nM mibolerone (*Mib*) or vehicle control (ethanol). Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent the mean ± S.E. from at least three independent experiments, each performed in triplicate. Reporter activity in the absence of transactivator or ligand was arbitrarily set to 1. *p* values were derived using *t* tests. *C*, Cos-7 cells expressing parental or mutant plasmids illustrated in *A* were maintained in serum-free medium containing 1 nM mibolerone (*Mib*, synthetic androgen) or ethanol (vehicle control) before staining with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). Cytoplasmic localization resulting from the R617A/K618A mutation is denoted by *white arrows*. *D*, DU145 cells were transiently transfected with 4XARE-E4-LUC and parental/mutant truncated AR variants exactly as in *B*. *E*, Cos-7 cells were transfected with parental/mutant truncated AR variants, stained, and imaged exactly as in *C*. Increased cytoplasmic localization resulting from the CE3 R617A/K618A mutation is denoted by *white arrows*.

androgen-independent growth. This observation is reminiscent of the biphasic growth response of LNCaP cells wherein androgen concentrations in the 0.1–1 nM range induce proliferation, but higher doses are growth-suppressive despite robust induction of AR target genes (30–32). Indeed, LNCaP cells transduced with lentivirus encoding AR 1/2/3/CE3 displayed a dose-dependent increase in expression of PSA and TMPRSS2 but were growth-suppressed at a high expression level (Fig. 9, *B* and *C*). This growth suppression was also observed when lentivirus-infected LNCaP cells were assayed the presence of androgens (see supplemental Fig. 8*A*). Moreover, under these conditions the AR 1/2/3/CE3 variant appeared to inhibit dihydrotestosterone-induced AR transcriptional activity perhaps via competition for ARE binding or feedback inhibition of full-length AR expression (33) (see supplemental Fig. 8*B*). In similar dose-response experiments with AR 1/2/3/2b or AR v567es, androgen-independent growth of LNCaP cells was apparent at lower expression levels, but higher levels of expression blocked this effect (see supplemental Fig. 8, *C* and *D*). Together, these data indicate that truncated AR variants can support androgen-independent PCa cell growth regardless of nuclear localization efficiency. To test this we performed AR knockdown/re-expression experiments in the 22Rv1 cell line (Fig. 9, *D* and *E*). Knocking-down AR expression using an exon 1-targeted siRNA inhibited androgen-independent growth, which was fully restored by lentiviral re-expression of siRNA-resistant AR 1/2/3/CE3 (Fig. 9, *D* and *E*). The nuclear/cytoplasmic AR 1/2/3/CE3 K629A/R631A mutant was also able to effectively

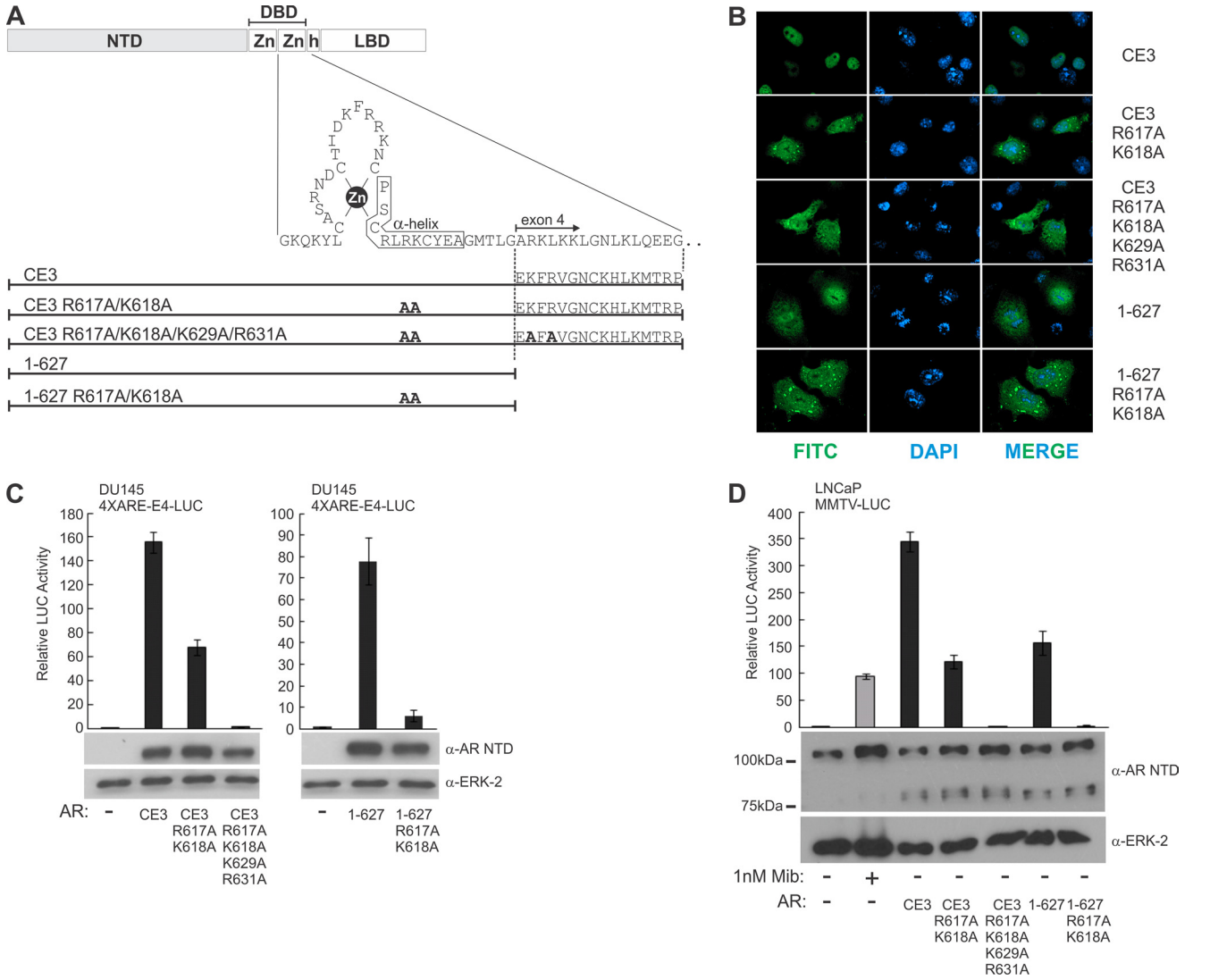


FIGURE 7. **The AR NTD/DBD core is sufficient for nuclear localization and transcriptional activity of truncated AR variants.** *A*, shown is a schematic of COOH-terminal deletions and alanine substitution mutations relative to key structural motifs in the second zinc finger of the AR DBD and hinge region. Zinc finger and α-helix locations were adapted from a prior depiction of the AR DBD crystal structure (46). *B*, Cos-7 cells expressing constructs depicted in *A* were maintained in serum-free medium and stained with an antibody specific for the AR NTD (FITC signal). Nuclei were stained with DAPI. Stained cells were visualized by confocal microscopy, and representative images are shown for three color channels (FITC, DAPI, and merged FITC/DAPI). *C*, DU145 cells were transiently transfected with 4XARE-E4-LUC and constructs depicted in *A*. Cell lysates were analyzed by luciferase assay and Western blot with antibodies specific for the AR NTD and ERK-2 (loading control). *Bars* represent mean ± S.E. from at least three independent experiments, each performed in triplicate. *D*, LNCaP cells were transiently transfected with MMTV-LUC and constructs depicted in *A*. Luciferase activity and transgene expression were assessed exactly as described in *C*. *Mib*, mibolerone.

restore androgen-independent growth, but the AR 1/2/3/CE3 R617A/K618A mutant displayed impairment (Fig. 9, *D* and *E*), which is consistent with the effects of these mutations on truncated AR variant transcriptional activity (Fig. 8, *A* and *B*).

In summary, our data demonstrate that a subset of truncated AR variants display enhanced nuclear localization due to reconstitution of classical NLS activity. However, a high, basal level of nuclear localization is mediated by the AR NTD/DBD core encoded by AR exons 1, 2, and 3. This basal level of nuclear localization is sufficient for strong, androgen-independent transcriptional activation of AR target genes and induction of androgen-independent growth. Therefore, truncated AR variants with diverse COOH-terminal extensions can contribute to CRPCa pathology by driving persistent AR activity during ADT.

DISCUSSION

Steroid receptors have extensive similarities in DBD and LBD structure, and a wealth of mechanistic information exists for steps leading to transcriptional activation. A key requirement for transcriptional activity of steroid receptors is interaction of unliganded receptors with the Hsp90 chaperone machinery. This interaction occurs exclusively through the LBD via direct binding to Hsp40 and Hsp70 (13, 34, 35). The Hsp90 chaperone complex association maintains AR in a conformation that inhibits DNA binding (36) and has high affinity for androgens (37). Chaperone release from steroid receptors is ligand-dependent, which results in a conformational change that exposes the bipartite AR NLS (RKCYEAGMTLGARKLKK) in the flexible hinge region between the AR DBD and

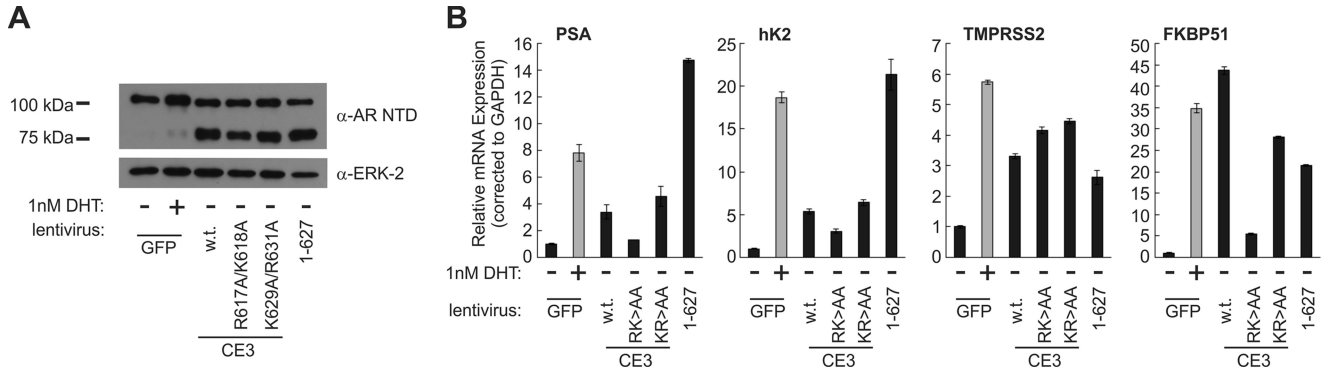


FIGURE 8. **The AR NTD/DBD core is sufficient for transcriptional activation of endogenous AR target genes.** *A*, LNCaP cells were transduced with lentivirus encoding GFP, parental and mutant versions of AR 1/2/3/CE3, or AR 1–627. Transduced cells were maintained under serum-free conditions and stimulated with 1 nM dihydrotestosterone (*DHT*) or vehicle control (ethanol) for 24 h before Western blot analysis with antibodies specific for the AR NTD or ERK-2 (loading control). *B*, LNCaP cells were transduced exactly as in *A*, and RNA was subjected to quantitative RT-PCR using primer sets specific for GAPDH, PSA, hK2, TMPRSS2, and FKBP51. Expression is shown relative to GAPDH as determined using the formula $2^{-\Delta\Delta Ct}$. *Bars* represent the mean ± S.D. from a triplicate experiment representative of three biological replicates.

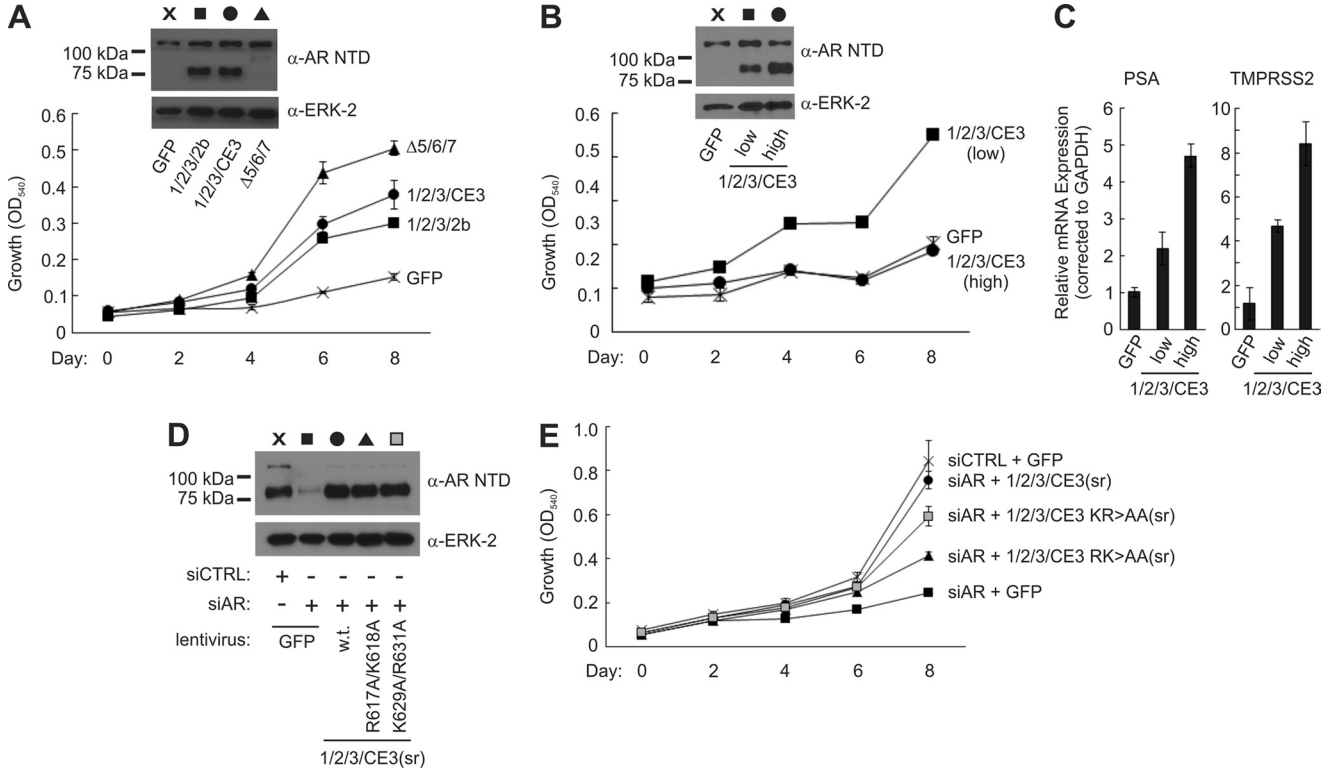


FIGURE 9. **Truncated AR variants support biphasic, androgen-independent growth of PCa cells.** *A*, LNCaP cells transduced with lentivirus encoding AR 1/2/3/2b, AR 1/2/3/CE3, or AR v567es were maintained in medium containing 10% charcoal-stripped (steroid-depleted) serum (10% charcoal-stripped serum). *Inset*, transduced cell lysates were analyzed by Western blot with antibodies specific for the AR NTD or ERK-2 (loading control). *Graph*, transduced cells were seeded on 24-well plates in medium containing 10% charcoal-stripped serum. At the indicated time points, cells were fixed and stained with crystal violet. Intensity of crystal violet staining (A_{540}) was used as a surrogate of cell number. Data represent the mean ± S.D. from a quadruplicate experiment representative of three biological replicates. *B*, LNCaP cells were transduced with increasing doses of lentivirus encoding AR 1/2/3/CE3 and subjected to Western blot (*inset*) and growth assay (*graph*) exactly as in *A*. *C*, LNCaP cells transduced as in *B* were subjected to quantitative RT-PCR using primer sets specific for GAPDH, PSA, and TMPRSS2. Expression is shown relative to GAPDH as determined using the formula $2^{-\Delta\Delta Ct}$. *Bars* represent the mean ± S.D. from a triplicate experiment representative of three biological replicates. *D*, 22Rv1 cells were transduced with lentivirus encoding GFP or siRNA-resistant forms (denoted *sr* (17)) of AR 1/2/3/CE3, AR 1/2/3/CE3 R617A/K618A, or AR 1/2/3/CE3 K629A/R631A and electroporated with siRNA targeted to AR exon 1 as indicated. Transduced/electroporated cells were subjected to Western blot with antibodies specific for the AR NTD or ERK-2 (loading control). *E*, 22Rv1 cells transduced/electroporated as in *D* were seeded on 24-well plates in medium containing 10% charcoal-stripped serum. At the indicated time points, cells were fixed and stained with crystal violet. Intensity of crystal violet staining (A_{540}) was used as a surrogate of cell number. Data represent the mean ± S.D. from a quadruplicate experiment representative of two biological replicates.

LBD. The crystal structure of the AR NLS peptide in complex with importin-α has revealed that the second cluster of basic amino acids (RKLKK), which is encoded by AR exon 4, engages with the major NLS binding site in importin-α (15). Interestingly, data presented here demonstrate that truncated AR variants display a constitutive, basal level of nuclear localization sufficient for ligand-independent transcriptional activity regardless of whether they harbor the exon 4-encoded NLS or NLS-like COOH-terminal extensions. Moreover, we have demonstrated that truncated AR variants access the nucleus inde-

pendently of the Hsp90 chaperone complex. Therefore, the signals that are critical for the regulatory cycle of the full-length AR appear to be dispensable for truncated AR variants.

It is well established that removal of the AR LBD results in ligand-independent AR transcriptional activity mediated by the AR NTD, but these early studies involved the use of constructs with deletions after the hinge region to retain the complete AR NLS (22, 38). The straightforward idea that NLS-mediated nuclear localization and transcriptional activity are intertwined has also led to controversy over the significance of many truncated AR variants that have been identified to date. For example, it has been postulated that truncated AR variants retaining the exon 4-encoded AR NLS would be functional transcription factors, whereas others would be transcriptionally incompetent (9, 10). This concept was supported by the demonstration that individual truncated AR variants display varying degrees of transcriptional activity (7) and an apparent correlation between the extent of nuclear localization and transcriptional activation (10). However, the current study demonstrates that transcriptional strength of individual truncated AR variants is a promoter-dependent phenomenon and independent of the magnitude of subcellular localization. Indeed, point mutations that inhibited the constitutive nuclear localization of the AR 1/2/3/CE3 isoform did not inhibit transcriptional activity. Similarly, point mutations that enhanced the nuclear localization of the AR 1/2/3/CE2 variant did not increase transcriptional activity. Even a synthetic truncated AR variant (1–627) that completely lacked the importin-α binding site (15) was transcriptionally active in a ligand-independent manner. Because these mechanistic principles were independent of promoter or cell line and were also extended to clinically relevant endogenous AR target genes, we conclude that constitutive transcriptional activity is likely an inherent property of truncated AR variants retaining the NTD/DBD core encoded by AR exons 1, 2, and 3. Our data also demonstrate that truncated AR variants with this NTD/DBD core can support androgen-independent growth when expressed in PCa cells. However, we have also shown that individual truncated AR variants need to be evaluated for growth-promoting effects very cautiously, especially in LNCaP cells, because the constitutive, androgen-independent transcriptional activity appears to induce a biphasic growth response in a fashion similar to high-dose androgen treatment (30–32). The molecular basis for the biphasic androgen effect is largely unknown but may arise from AR-mediated activation of differentiation-promoting genes or AR-mediated transcriptional inhibition of the AR promoter at higher androgen concentrations (33).

If the canonical nuclear localization signal is dispensable for truncated AR variant function, then what are the mechanisms that promote their entry into the nucleus? A previous live cell imaging study with fluorescence-labeled AR fragments demonstrated that the AR NTD has strong nuclear import activity in isolation (39). Moreover, deletion of the RKLKK motif in full-length AR has been shown to impair nuclear localization but induces a paradoxical superactivity in response to androgens (40), which may be due to enhanced intranuclear mobility (41). However, studies where the AR NLS has been manipulated by mutation and/or deletion have historically been difficult to interpret because lysine residues in the AR NLS are also acetylation targets (42, 43). The fact that the AR 1–627 fragment employed in this study could access the nucleus and activate endogenous AR target genes with high efficiency clearly confirms that functional nuclear targeting signals exist outside of the AR hinge/LBD region. Based on these findings, we conclude that the canonical NLS in the AR hinge region is not the only determinant of AR nuclear access and transcriptional activity in PCa cells.

Intriguingly, a previous study demonstrated that androgen-independent growth induced by truncated AR variants required full-length AR (10). One explanation for this phenomenon could be a physical interaction between full-length AR and truncated AR variants, which is supported by *in vitro* experiments with overexpressed full-length AR and AR v567es (9). However, we have recently demonstrated that the LuCaP 86.2 xenograft, where the AR v567es variant was discovered, harbors a 8.5-kb deletion of AR exons 5, 6, and 7 in the sole AR gene copy at Xq11–12 (44). This would preclude a scenario where full-length AR and AR v567es could co-exist in the same cell in this model. Similarly, in this and other studies we have demonstrated that knockdown of full-length AR in the 22Rv1 and CWR-R1 models of CRPCa has no effect on androgen-independent expression of AR target genes or androgen-independent growth. Conversely, knock-down of endogenously expressed truncated AR variants inhibits both of these parameters (28, 44). These data strongly support the concept that truncated AR variants possess the biochemical properties required to independently support ongoing PCa cell growth during ADT. Indeed, PCa cells with AR intragenic rearrangements and altered splicing patterns have a competitive growth advantage under castrate conditions (28, 44). The finding that intragenic copy number imbalances are frequent in CRPCa (44) portends complex and diverse AR splicing patterns in human tumors, which is also supported by unbiased tiling array studies (27). Antibody development efforts in this and previous studies show that rearrangement-dependent splicing alterations do indeed give rise to translated, functional proteins such as AR 1/2/3/2b and AR 1/2/3/CE3 (7, 8). Our data establish new biochemical principles that will be critical for studying the role of known as well as yet-to-be-discovered truncated AR variant species in PCa progression.

Our data also highlight the potential application of targeting truncated AR variants for therapy of CRPCa. For example, inhibitors such as EPI-001, a bisphenol A-derivative that appears to interfere with the function of transcriptional activation domains in the AR NTD (45), would be anticipated to block transcriptional activity and growth-supporting functions of truncated AR variants. Our data also show that truncated AR variants have a unique requirement for structural integrity of the second α-helix in the AR DBD, which might be an additional focal point for inhibitory strategies. We have further shown that higher levels of overexpression of the truncated AR 1/2/3/CE3 variant in LNCaP cells leads to paradoxical lower levels of cell growth, especially under conditions where androgens are present. Androgen-induced expression of AR target genes was also reduced after expression of the truncated AR 1/2/3/CE3 variant. This indicates that targeted inhibition of

truncated AR variants may be most effective when activity of full-length AR is durably suppressed. However, it is also possible that these results may reflect competition between ligand-bound AR and truncated AR variants for ARE binding and/or an acute sensitivity of LNCaP cells to excessive levels of AR signaling. Further investigation in additional models is required to fully understand the potential application and optimal conditions for targeting truncated AR variants in CRPCa.

In summary, our data demonstrate that truncated AR variants with the NTD/DBD core are constitutively active, ligand-independent transcription factors that can support androgen-independent growth of PCa cells. Expression of truncated AR variants is frequent in CRPCa metastases and associated with poor prognosis (11). Therefore, AR variant species with the NTD/DBD core should be viewed as key therapeutic targets at this stage of the disease.

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